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(57) Abstract

In accordance with the invention, pharmacologically effective glucocorticoids can be delivered to a targeted cell population using a protein carrier molecule that has a binding site for glucocorticoids and a binding site specific to the targeted cell population. Such carrier molecules may be derived from human corticosteroid binding globulin (hCBG) by using a recombinant plasmid expressed in insect or other eukaryotic cells to produce a chimeric protein comprising an active steroid-biding moiety derived from hCBG and a cell binding moiety having a peptide sequence selected to specifically recognize and bind to the target cell population. For example, portions of the peptide gp120 of human immunodeficiency virus (HIV) may be used to bring about selective steroid delivery to the HIV target cells, CD4+ lymphocytes.

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Description

Steroid Hormone Drug Delivery

Specification

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Background of the Invention

This invention relates to cell specific steroid hormone delivery utilizing a recombinant heterologous protein carrier molecule.

Administration of glucocorticoids is one of the more effective treatments for arthritis and other inflammatory diseases, but it is also one of the more toxic modalities. This is presumably due to the fact that most cells in the body respond to glucocorticoids, and under normal conditions the levels of glucocorticoids in the circulation and in peripheral tissues are carefully regulated. Corticosteroid binding globulin (CBG) is the major serum binding protein for cortisol involved in this regulation; greater than 80% of the circulating cortisol is bound to this protein, the remainder being primarily associated with serum albumin.

Although it has been generally thought that steroids enter target tissues by passive diffusion, recent evidence strongly suggests that CBG delivers corticosteroids to target cells by binding to membrane receptors. Siiteri et al., Rec. Prog. Horm. Res., 38:457 (1982); and Kuhn, Ann. New York Acad. Sci., 538:146 (1988). In addition, it has been proposed that at sites of inflammation, limited proteolysis of CBG

results in an enhanced release of bound steroids. Pemberton et al., Nature, 336:257 (1988); and Smith and Hammond, abstr. 22 71st Ann. Meeting Endocrine Society, June (1989). It is an object of the present invention to manipulate the interaction of CBG with target cells (using a molecular genetic approach), to form a basis for controlling glucocorticoid pharmacological action, and provide novel therapeutic agents and drug delivery molecules.

Summary of the Invention

In accordance with the invention, pharmacologically effective glucocorticoids can be delivered to a targeted cell population using a protein carrier molecule that has a binding site for glucocorticoids and a binding site specific to the targeted cell population. Such carrier molecules may be derived from human corticosteroid binding globulin (hCBG) by using a recombinant DNA expressed in insect or other eukaryotic cells to produce a chimeric protein comprising an active steroid-binding moiety derived from hCBG and a cell binding moiety having a peptide sequence selected to specifically recognize and bind to the target cell population. For example, portions of the peptide gp120 of human immunodeficiency virus (HIV) may be used to bring about selective steroid delivery to the HIV target cells, $CD4^+$ lymphocytes.

Brief Description of the Drawing

Figure 1 is a graph showing the immunoquantitation of hCBG production in the presence of various factors. The solid circles (•) represent purified human serum hCBG; the solid triangles (▲) represent human serum and the open circles (o) represent *sf9* cell lysate.

Detailed Description of the Invention

In accordance with the present invention, corticosteroid binding globulin (CBG) is expressed in insect or other eukaryotic cells, either in the form of active CBG per se, active steroid-binding fragments of CBG, chimeric proteins of CBG, or active fragments thereof with other proteins. In particular, the invention relates to chimeric proteins in which an active steroid-binding moiety derived from CBG is coupled to a cell binding moiety having a heterologous peptide sequence effective to specifically recognize and bind to a target cell population. In particular, the target cell population can be different from the target cell population of native CBG.

As used herein, a peptide sequence which is "derived from" a particular source is a sequence which corresponds sufficiently to the sequence of the particular source so as to substantially retain the function of the source peptide sequence. It will be understood, however, that variations in peptide sequence can in many cases be made without loss of function, particularly if the variations occur at a residue remote from the active site. Protein sequences derived from the particular source encompass all such sequences, however made. For example, protein sequences can be made by expressing a cloned DNA fragment encoding the protein obtained from a naturally occurring sample, or can be derived from a synthetic peptide. The DNA fragment may be obtained by any method known in the art for instance by direct cloning of genomic DNA, by synthesis of complementary DNA (cDNA) or by amplification of genomic DNA, cDNA or messenger RNA (mRNA) by methods such as polymerase chain reaction (PCR).

For some purposes, it is convenient to have available a supply of recombinant CBG which has not

been modified. For example, recombinant native human CBG (hCBG) may be useful in the treatment of arthritis and other inflammatory diseases. In other cases, heterologous cell binding moieties are desirable to target steroid delivery to other specific cell types. Such target cell populations include $CD4^+$ lymphocytes, which may be targeted using the cell binding portions of the gp120 peptide of HIV, or any lymphocyte type cell containing cell surface receptors which bind specific peptide ligands. Thus, the invention encompasses both recombinant CBG and chimeric proteins of CBG with heterologous cell binding moieties.

Recombinant CBG or CBG-chimeric proteins of the invention can be used to deliver corticosteroids to specific target cells of a patient. In this case a substantially purified preparation of the appropriate CBG or CBG chimeric protein having corticosteroid bound to it is administered (e.g., intravenously, subcutaneously) to the patient. For these purposes, the term "substantially purified" means sufficiently separated from other proteins and toxins and the like to avoid unacceptable adverse reactions.

In order to make use of hCBG as a starting point for the construction of chimeric proteins in accordance with the invention, it is necessary to determine several things about CBG, i.e., how CBG acquires its native structure in vivo, the kinetics and thermodynamics of steroid binding and what portion of the CBG sequence is involved in steroid binding. With this knowledge, it is possible to construct heterologous chimeric proteins containing the steroid-binding domain of CBG and a cell-type specific cell binding moiety.

A. Acquisition of Native Structure

The existence of CBG as a protein having high affinity for corticosteroids was first demonstrated in

1956 and again, independently, in 1957. Daughaday, J. Lab. Clin. Med., 48:799 (1956); and Bush, CIBA Found. Colloq. Endocrinol., 7:263, Little Brown & Co. (1957). hCBG is a monomeric glycoprotein with an apparent molecular weight of 45 - 50 kD containing about 30% (w/w) N-linked carbohydrate, and one steroid-binding site. CBG has high affinity for cortisol and corticosterone, but relatively low affinity for dexamethasone. Westphal, Steroid-Protein Interactions II, Springer-Verlag, Berlin, Heidelberg, New York (1986).

The DNA sequence encoding hCBG predicts a protein of 405 amino acids, containing an amino-terminal signal peptide that targets the nascent protein to the secretory pathway, as well as six potential sites for N-linked glycosylation. Hammond et al., Proc. Natl. Acad. Sci. (USA), 84:5153 (1987). This prediction has been confirmed by the synthesis of CBG described herein in a cell-free system and expression in insect cells.

The presence of these molecular signals within the sequence of CBG implies that in intact cells, CBG undergoes a complex series of sorting and processing events from the time of its synthesis to its secretion in a biologically active form. Its amino-terminal signal peptide targets it to the rough endoplasmic reticulum (RER) membrane, whereupon it is translocated into the RER lumenal space. Subsequently, it most likely undergoes sequential signal peptide cleavage and core glycosylation in the RER, processing and maturation of its oligosaccharide moieties in the Golgi apparatus, packaging into post-Golgi secretory vesicles, and finally release from the cell by exocytosis. These phenomena are normally associated with mammalian host systems, but are lacking in bacterial hosts. Unfortunately, mammalian cells require the presence of cortisol for growth which

complicates the production of a cortisol binding protein in such a system.

It has now been found that biologically functional hCBG can be expressed in *Spodoptera frugiperda (sf9)* cells thus obviating the need for excess cortisol. hCBG is expressed in *sf9* cells after infection with a recombinant baculovirus derived from mixed transfection with baculovirus (AcMNPV) DNA, and a plasmid derived from pVL-1393 (Invitrogen) containing the hCBG gene under the control of the baculovirus polyhedrin promoter, (See Example 5), although other vectors which include a promoter recognized by insect cells could also be used. Other host cells which may be used include but are not limited to Chinese hamster ovary (CHO) cells.

B. Determination of Steroid Binding Site

The amino acid sequence of CBG also suggests that it belongs to the SERPIN super-family, which includes α-1-antitrypsin and thyroxine binding globulin. Bush, CIBA Found. Colloq. Endocrinol., 7:263, Little Brown & Co. (1957). On the basis of the sequence homology to α-1-antitrypsin, it has been suggested that proteolytic cleavage at a specific elastase-sensitive sequence facilitates the release of corticosteroids from CBG at sites of inflammation. Pemberton et al. (1988). Direct evidence for this specific cleavage of hCBG has recently been reported. Smith and Hammond (1989).

There is evidence from changes in the absorption and fluorescence spectra of several steroid-binding proteins, including CBG, that aromatic amino acid residues play an important role in steroid-binding. For example, the tryptophan fluorescence properties of serum albumin, progesterone binding globulin, and CBG are perturbed by steroid binding. Atallah and Lata, Biochem. Biophys. Acta, 168:321 (1968); Ryan and Gibbs,

Arch. Biochem. Biophys., 136:65 (1970); Stroupe et al., FEBS Lett, 86:61 (1978); and Stroupe et al., Biochemistry, 17:177 (1978). In the case of hCBG, the tryptophan fluorescence is quenched by bound steroid. (See Example 2). This suggests that at least one of the four tryptophan residues present in the sequence of hCBG is at or near the steroid binding site.

It also appears that at least one cysteine residue plays a role in the CBG binding site for steroids since interaction of hCBG with cortisol protects one of the two cysteine residues from chemical modification. Defaye et al., Biochem. Biophys Acta, 623:280-294 (1980).

Using the expression system disclosed herein, site directed mutagenesis can be used to determine which of the cysteine residues is protected, and which of the tryptophan residues are involved. It is noteworthy that Cys_{228} and all four tryptophan residues are conserved in human, rat and rabbit CBG protein sequences indicating that these amino acid residues are functionally significant. Hammond et al. (1987); Smith and Hammond (1989); and Seralini et al., Mol. Endocrinol., 4:1166-1172 (1990).

Based upon the available information, the critical part of the CBG sequence necessary to achieve steroid binding includes amino acid residues 228 through 371 of CBG. Thus, the DNA sequences encoding this portion of the protein sequence may be fused to heterologous DNA sequences encoding cell binding protein moieties to create chimeric proteins in accordance with the invention.

Chimeric bifunctional proteins formed from at least the steroid binding portion of CBG and a peptide selected to bind to specific target cells have the potential to deliver a variety of steroids directly to any number of specific target cells. The success of

this approach depends upon the choice of cell-specific peptide ligands which will bind to their receptors when part of a CBG chimeric protein. The decrease in steroid binding below pH 7.4 suggests that internalization of chimeric proteins via receptor-mediated endocytosis results in the release of bound steroid in an endosomal compartment, where the bound steroid would be free to diffuse into the cytoplasm of the target cell. If steroid dissociation does not occur in the endosomal compartment, subsequent delivery of the protein-steroid complex to the lysosome results in steroid release by proteolytic degradation of the protein.

An embodiment of the present invention is recombinant cDNA that encodes a chimeric protein between CBG or an active steroid binding portion thereof and the gp120 envelope glycoprotein of human immunodeficiency virus (HIV).

Infection of T lymphocytes by HIV has been shown to involve direct binding of gp120 to the $CD4^+$ cell surface receptor. Kowalski et al., Science, 237:1351 (1987); Lasky et al., Cell, 50:975 (1987); and Sattentau and Weiss, Cell, 52:631 (1988).

In the experiments described herein, gp120 was selected as the cell receptor binding portion of the chimeric protein to facilitate the delivery of therapeutic steroids to HIV infected T-lymphocytes which are particularly susceptible to the cytotoxic action of glucocorticoids.

The present invention also encompasses other embodiments wherein the cell-targeting domain of the chimeric molecule is any protein, peptide or protein segment capable of binding to a particular cell type or types with reasonable specificity without interfering with the ability of CBG to bind steroid hormones. For example, a chimeric protein between CBG and residues

68-88 of myelin basic protein (MBP) may be effective in treating experimental allergic encephalitis in Lewis rats. This disease, which is considered to be an animal model for human autoimmune diseases such as multiple sclerosis, results in the production of B- and T- lymphocytes in the central nervous system that recognize epitopes of MBP. Thus, the present invention provides that administration of CBG-MBP chimeric proteins will result in targeting of the chimeric proteins to specific lymphocytes in such animals. The invention also encompasses mutant CBG molecules or portions thereof capable of differentially binding steroid hormones.

The chimeric CBG/gp120 molecule of the present invention is constructed as follows. The gp120 protein of HIV consists of 511 amino acids. The amino acid residues responsible for interaction with the CD4 protein have been mapped to the carboxy-terminal 148 amino acid residues of gp120. Kowalski et al., (1987); Lasky et al., (1987); and Sattentau and Weiss, (1988). The DNA sequence encoding gp120 (the "gp120 DNA sequence") is fused at either the 3′ or 5′ end of the DNA sequence encoding CBG (the "CBG DNA sequence"). For example, the DNA sequence encoding residues 363-511 of gp120 may be fused in-frame to the 3′ terminus of the appropriate CBG DNA sequence (CBG/gp120). In an alternate construction, the identical gp120 DNA sequence is inserted 5′ to the mature CBG DNA sequence, retaining the upstream initiator methionine codon and the DNA sequence encoding the CBG signal peptide (gp120/CBG). Thus, expression of the chimeric genes results in chimeric proteins wherein the CD4-binding domain of gp120 is either amino-terminal or carboxy-terminal to the steroid-binding domain of CBG. The fusion genes are cloned into a suitable expression vector and transferred into a suitable expression

system. The chimeric proteins are then produced in a suitable system such as the baculovirus system, and prior to use are assayed for 1) steroid binding properties, and 2) acquisition of CD4 binding capability.

The ability of the chimeric proteins of this type to target steroid-responsive cells, can be determined using several human lymphoma cell lines to monitor uptake and activity of the chimeric proteins: CEM and Jurkat cells, which are strongly and uniformly CD4 positive; and HCD8 and Raji which serve as the corresponding CD4 negative control cell lines. The above-mentioned cells are incubated in defined growth medium in the presence of CBG, CBG/gp120, or gp120/CBG which have been complexed with corticosteroid. CBG alone is not recognized by the CD4 cell surface receptor. As further controls, cells are incubated with steroid-free proteins, and corticosteroid in the absence of binding proteins. Binding and uptake of the chimeric proteins are monitored by the use of [^{35}S]-labelled chimeric proteins, as well as [^{3}H]-cortisol. Most importantly, the viability of cells treated with the chimeric protein-cortisol complexes is assessed by fluorometric assay of propridium iodide uptake as uptake of cortisol via CD4-mediated endocytosis results in cell death in a dose-dependent manner.

In vivo expression systems in accordance with the invention provide the ability to produce metabolically radiolabelled CBG of high specific activity. Radiolabelled material may be used to probe binding of CBG to specific cell surface receptors on target cells. Clearly, metabolically-labelled CBG is superior to the currently used chemically radiolabelled CBG.

The following examples are meant to illustrate but not limit the present invention. Unless specified otherwise, the molecular biology techniques used in the

Examples are those described in Sambrook et al., "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Press. NY (1990).

Example 1

Purification of CBG

Several methods for purification of human hCBG have been published. Westphal, in Steroid-Protein Interactions II, Springer, New York, Vol. 29 (1986). The procedure used herein was according to the procedure described by Westphal, Biochemistry, 18:2685 (1979); Mickelson et al., Biochemistry, 20:6211 (1981); and Mickelson et al., Biochemistry, 21:654 (1982), which involves affinity chromatography with 11-hydroxy-3-oxo-4-androstene-17-carboxy-aminoethylamine-1,4-butanediol diglycidyl ether-agarose. This affinity matrix, which is referred to here as the cortisol affinity resin, is reported to have superior stability because of the ether linkage. Since guinea pig CBG can be purified by the same procedure as hCBG, and since both proteins exhibit similar changes in intrinsic fluorescence upon binding steroids, the guinea pig protein has been used as a model system. Westphal (1986). Using the cortisol affinity resin, guinea pig CBG was purified for model fluorescence studies, as described below.

These same purification techniques can be used to purify recombinant CBG or CBG-chimeric proteins.

Example 2

Intrinsic Tryptophan Fluorescence of CBG

The tryptophan fluorescence of CBG is quenched by steroid binding. One possible quenching mechanism is perturbation due to a conformational change as a result of steroid binding. Another possible quenching mechanism is resonance energy transfer from the excited state of tryptophan to the steroid 3-oxo-4-enechromophore. The near-ultraviolet absorption band

of the 3-oxo-4-ene chromophore is a weak $n \rightarrow \pi^*$ transition which overlaps with the tryptophan fluorescence emission spectrum. This would suggest that the indole ring and steroid chromophore are close together; to satisfy the conditions for resonance energy transfer, the tryptophan(s) must be within a few angstroms of the bound steroid.

The tryptophan indole ring could participate in a stacking interaction with the steroid because it has a large, rigid, aromatic surface which allows stable, short-range van der Waals interactions, and it has a large ground-state dipole moment which provides long-range, mutual orientation interactions with other dipoles. In addition, the hydrogen atoms of the indole nitrogen could participate in hydrogen bonds with the bound steroid.

The effects of cortisol and 5-pregnane-3,20-dione binding upon the tryptophan fluorescence of guinea pig CBG have now been measured; the latter steroid lacks the 3-oxo-4-ene chromophore and binds with about 30% the affinity of cortisol. In agreement with Mickelson et al. (1981), whereas cortisol binding results in quenching of the tryptophan emission, 5-pregnane-3,20-dione binding results in blue-shift of the emission.

The quenching by cortisol binding is consistent with energy transfer to the 3-oxo-4-ene chromophore, and the blue-shift by 5-pregnane-3,20-dione binding is consistent with an exposed tryptophan being shielded from water. The latter conclusion has now been tested by collisional quenching of the tryptophan fluorescence with iodide and acrylamide. In the presence of 5-pregnane-3,20-dione, neither collisional quencher affects tryptophan emission. This result confirms the interpretation that when CBG is complexed with steroid, the emissive, blue-shifted tryptophan is not accessible to solvent. It also supports the hypothesis that the

quenching by cortisol is due to a close interaction with the tryptophan ring, and that the tryptophan is within the steroid-binding site.

When no steroid is bound, iodide, a negatively charged quencher, is a more effective quencher of the CBG tryptophan fluorescence than acrylamide. By contrast, acrylamide is a slightly better quencher of uncharged tryptophan model compounds. Since acrylamide is an uncharged, polar molecule, the latter result suggests that at neutral pH there is/are positively charged residue(s) near or in the steroid-binding site of guinea pig CBG. This is an important observation because the steroid-binding affinity of both guinea pig and hCBG is optimal between pH 8-9.

Example 3

Cloning of hCBG

A full-length cDNA encoding hCBG has been isolated for use in mutagenesis and expression studies. A human liver cDNA library that had been cloned into the restriction endonuclease *Eco*RI site of the bacteriophage λgt11 was screened. Briefly, *Escherichia coli* cells were infected with the phage library, plated on agar, and phage plaques (each derived from a single phage-infected *E. coli*) were transferred by blotting to duplicate nitrocellulose filters. After chemical treatment to expose and denature the phage DNA, the filters were hybridized individually with two [^{32}P]-labelled oligonucleotide probes, one corresponding to nucleotides 25-49 of the reported 5′ DNA sequence of hCBG, (5′-TATACTGGACAATGCCACTCCTCCT-3′) including the translation initiation codon, and the second, of identical length, corresponding to the 3′ DNA sequence that includes the TAA termination codon, nucleotides 1371-1395 (5′-CAGACTTGTGTCTAACTTTAGCCAT-3′). Plaques that hybridized strongly to both probes were isolated

(this implies that they contain the full-length cDNA encoding hCBG ("hCBG cDNA"), and recombinant phages were subjected to a secondary and tertiary screening. Finally, phage DNA was isolated and digested with *Eco*RI; producing a 1.4 kb fragment. By restriction endonuclease mapping and partial DNA-sequencing of the DNA fragment, it was established as encoding hCBG.

Example 4

Synthesis of CBG in vitro

The cDNA encoding hCBG has now been expressed in a cell-free system by subcloning the 1.4 kb *Eco*RI fragment (Example 3) into the pGEM1 plasmid vector according to the manufacturer's instructions (Stratagene, Promega CA). The pGEM1 vector was utilized in in vitro synthesis of hCBG mRNA, using T7 RNA polymerase. The hCBG mRNA thus synthesized was then used to program protein synthesis in the rabbit reticulocyte lysate translation system in the presence of [^{35}S]-Met according to the manufacturer's instructions (Promega). The protein products obtained were analyzed by subjecting them to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli, Nature, 227:680-685 (1970). A major radiolabelled hCBG-immunoreactive protein of 45 kD was produced. When canine pancreatic microsomes prepared by the method of Shields and Blobel, J. Biol. Chem., 253:3753-3756 (1978), were included in the translation reaction, several higher-molecular weight species were generated that correspond to CBGs containing several core carbohydrate moieties. Addition of trypsin to the translation reaction containing pancreatic microsomes resulted in degradation of the primary unglycosylated, translation products, while the glycosylated proteins were protected from proteolysis by the microsomal membranes indicating that the glycosylated proteins were

translocated across the microsomal membranes and thus not exposed to the trypsin.

Co-translational glycosylation was confirmed by subjecting the protected material to digestion with Endoglycosidase H prior to electrophoresis according to the manufacturer's instructions (Genzyme). Such digestion selectively removes the endoplasmic reticulum-derived (ER) N-linked carbohydrate. Endoglycosidase H digestion resulted in the disappearance of the membrane-derived species and the appearance of a glycosylated form which was of slightly lower apparent molecular weight than the primary translation product. This behavior is consistent with appropriate cleavage of the amino-terminal signal peptide. These experiments demonstrate that the amino-terminal sequence of (pre)-CBG functions as a canonical signal peptide to mediate co-translational uptake of CBG into the secretory pathway.

Example 5

CBG Expression in vivo

Expression of heterologous proteins in the baculovirus system takes advantage of the fact that virally infected insect cells synthesize high levels of a non-structural viral-encoded protein, the polyhedrin protein. Lukow and Summers, Bio. Tech., 6:47 (1988); Jarvis and Summers, Mol. Cell Bio., 9:214 (1989); and Lukow and Summers, Virology, 170:31 (1989). Since, under certain conditions, viral infection proceeds even in the absence of the polyhedrin protein, it is possible to replace the DNA encoding the polyhedrin protein ("polyhedrin DNA sequence") with a foreign DNA sequence (e.g., a CBG DNA sequence). The expression of the foreign DNA sequence is thus under control of the polyhedrin promoter. Such recombinant viruses can then be used to infect target *sf9* insect cells, which then

synthesize large amounts (up to 10% of the total cellular protein) of the foreign protein.

Construction of recombinant virus was carried out in two steps. First, hCBG cDNA was cloned into the pVL1393 plasmid according to the method described by Lukow and Summers (1989). pVL1393 contains the polyhedrin promoter DNA sequence followed by an *Eco*R1 insertion site. The recombinant plasmid was then introduced into *sf9* cells along with intact baculovirus DNA. This allowed DNA recombination events to occur that replace the polyhedrin DNA sequence in the viral DNA with that of the CBG DNA sequence, while retaining the polyhedrin promoter. Detection and purification of the recombinant viruses was accomplished by transferring viral plaques to nitrocellulose filters, followed by hybridization with a radiolabelled CBG cDNA probe as described for the bacteriophage library by Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, New York (1982). After several cycles of plaque purification, the recombinant viruses were used to infect *sf9* cells at high multiplicity for production of CBG.

Two days after viral infection, *sf9* cells were radiolabelled with [^{35}S]-Met for 2h, and cell lysates and media were subjected to immunoprecipitation with anti-hCBG antibodies, followed by resolution on SDS-PAGE. A CBG-immunoreactive polypeptide of 55 kD was detected that was present only in cells infected with CBG-recombinant virus, and absent in uninfected cells or cells infected with wild-type baculovirus. The 55 kD polypeptide is glycosylated, since pretreatment of cells with tunicamycin to inhibit glycosylation resulted in the disappearance of this species and appearance of a lower molecular weight form corresponding in size to unglycosylated CBG. In addition, treatment of this material with

Endoglycosidase H resulted in a partial depletion of the 55 kD species, with a concomitant increase in the unglycosylated form that corresponds to the signal peptide cleaved species.

Secretion of hCBG from *sf9* cells was assessed by parallel immunoprecipitations of the labelling medium. A 55 kD species was observed that co-migrated with the major intracellular band. Under these conditions, at least 50% of the newly synthesized CBG is secreted by the cells.

These data indicate that insect cells can synthesize and secrete hCBG.

Example 6

Biological Activity of Recombinant hCBG

The steroid-binding properties of hCBG derived from *sf9* cells and human serum were compared using saturation binding and Scatchard analyses according to the method described by Schiller and Petra, J. Steroid Biochem., 7:55-59 (1976); and Ross et al., FEBS Letts. 149:240-244 (1982). The determination of active hCBG in human serum by saturation binding of [^{3}H]-cortisol correlates well with the amount measured by immunoassay (Table 1).

TABLE 1

Production of Recombinant hCBG

	human serum	*sf9* lysate	*sf9* medium
IR (pmol)	50	113	60
^{3}H-Cs (pmol)	54	6	48
RBA	1.08	0.05	0.80

In Table 1, Values are for 100 μl of human pregnancy serum, and for lysate and medium fractions derived from 2.5 x 10^6 *sf9* cells. Immunoreactive hCBG

(IR) was quantified as follows. 1 x 10^5 cpm of [^{35}S]-Met-labelled translation products obtained as in Example 1, were incubated with sufficient polyclonal anti-hCBG antiserum to achieve 50% immunoprecipitation, in the presence of hCBG (•), human serum (▲) and *sf9* (○) cell lysate in the concentrations indicated in Figure 1. The displacement curve in Figure 1 of *sf9*-derived material is parallel to that of hCBG purified from human serum, indicating a high degree of similarity in antigenic determinants. To measure secretion, infected cultures were fed fresh medium at 48 hours post-infection, and cells and media were harvested at 90 hours. Under these conditions, 10^6 infected cells contain 2.3-3 μg (58-75 pmol) immunoreactive hCBG, and media derived from 10^6 cells contain 1 μg (25 pmol) hCBG (Table 1); this corresponds to the secretion of approximately 1-2 μg/ml/24h in monolayer culture. The immunoprecipitates were resolved on SDS-PAGE, and the amount of radiolabelled hCBG recovered in antigen-antibody complexes was quantified by densitometry. Saturation binding of [^{3}]H-cortisol (^{3}H-Cs) was measured as follows in the filter assay described by Schiller and Petra, J. Steroid Biochem., 7:55-59 (1976). Equilibrium association constants (Table 2) were determined by competition with unlabelled steroids according to the method described by Ross et al., FEBS Letts., 149:240-244 (1982). *sf9* cell lysates, human serum, and purified hCBG derived from human serum were assayed directly after dilution by densitometry. In 50 μl reactions, binding is linear between 25-250 fmol hCBG, and the standard error is ± 16%. The relative binding activity (RBA) is the ratio ^{3}H-Cs/IR.

The results presented in Table 1 indicate that, while uninfected *sf9* cells have negligible [^{3}H]-cortisol-binding activity, infected cultures contain

10-25 pmol per 10^6 cells. The secreted fraction contains 75-90% of the total activity, and this material binds 0.7-0.9 moles cortisol per mole immunoreactive hCBG (Table 1). Most importantly, the affinity of the secreted recombinant protein for cortisol is indistinguishable from that of serum hCBG, as is the rank order of binding of other steroids (Table 2).

TABLE 2

Comparison of Steroid Binding by Recombinant and Serum hCBG

	human serum		*sf9* cells	
Steroid	$K_a(M^{-1})$	$\frac{K_a}{K_aC_s}$	$K_a(M^{-1})$	$\frac{K_a}{K_aC_s}$
Cs	2.6×10^8	1.0	1.8×10^8	1.0
Prg	4.3×10^7	0.17	2.2×10^7	0.12
Ts	1.8×10^6	0.007	1.0×10^6	0.006
Dex	5.5×10^4	0.0002	2.6×10^4	0.0001
Ecd	$<1.4 \times 10^4$	--	$<1.4 \times 10^4$	--

In Table 2 the association constants for the other steroids were determined by their competition with [^{3}H]-cortisol at 50% saturation of binding. The error in the association constants is a factor of 2. The abbreviations used are: Cs, cortisol; Prg, progesterone; Ts, testosterone; Dex, dexamethasone; Ecd, ecdysone.

The data presented in Table 2 indicate that *sf9* cells secrete authentic hCBG that contains a native steroid-binding site.

Example 7

Effect of Glycosylation on Steroid-Binding Activity

To examine the role of glycosylation in the production of active hCBG, infected *sf9* cells were treated with tunicamycin, which inhibits Asn-linked glycosylation. Metabolic labelling revealed the synthesis of both hCBG containing the signal peptide (S+) and mature hCBG with the signal peptide cleaved (S-). As previously observed in hepatoma cells, unglycosylated hCBG is secreted at reduced levels relative to glycosylated forms of hCBG (GP-hCBG). Longer autoradiographic exposures also indicated residual synthesis of GP-hCBG in tunicamycin-treated cells; this was confirmed by immunoblot analysis of unlabelled media from parallel cultures. To separate S+ and S- hCBG from the glycosylated forms, this material was adsorbed to Concanavalin A-Sepharose. Saturation binding of [^{3}H]-cortisol to secreted hCBG was reduced to 24% of the control value by tunicamycin treatment, and Concanavalin A-purified unglycosylated hCBG was completely inactive.

Example 8

CBG Deletion Mutant Analysis

To synthesize CBG molecules with carboxyl-terminal deletions, CBG cDNA cloned into the pGEM plasmid is digested with restriction endonucleases that recognize sequences in the 3′ half of CBG cDNA, so as to remove a DNA fragment, and religated without the fragment. Alternatively, CBG DNA is cleaved with a restriction endonuclease, subjected to limited digestion with Bal31 nuclease, religated, and subcloned after linker addition. Production of CBGs with amino-terminal deletions is somewhat more complicated. To assure appropriate synthesis and secretion, there is a need to retain the 5′ untranslated sequence, the initiator

methionine codon, as well as the signal peptide DNA sequence. In these cases, oligonucleotide-directed site-specific deletion mutagenesis is performed (similar to the site-directed procedure described below), to generate precise internal deletions of the DNA sequence encoding the amino-terminus of the protein.

After manipulation, the truncated cDNAs are transcribed and translated in vitro. Cell-free synthesis is particularly useful in this case to screen for mutants that are abnormal in translocation and glycosylation and might not be detectable if translated in vivo. After analysis of a series of truncated in vitro translation products, the appropriate cDNAs are cloned, e.g. in pVL1393. The mutant proteins are produced in sufficient quantities for physical and chemical characterization.

Example 9

Site-Directed Mutants

The tryptophan residue(s) involved in steroid binding are specifically identified using tryptophan mutants prepared by site-directed mutagenesis. The strategy for mutagenesis is based upon the chemical and spectroscopic studies that have 1) established that a cysteine (Cys) residue is essential for steroid binding, and 2) implicated one or more tryptophan (Trp) residues as being in close proximity to the ligand-binding site. Westphal (1986). Since there are only two Cys and four Trp residues in the sequence of mature hCBG, each of these six residues, singly and in combination, is mutated in order to identify the relevant amino acids and investigate what role they might play in steroid binding. For example, isosteric substitution of one of the Cys residues with a Ser residue may eliminate steroid binding entirely. This result would pinpoint a critical residue and suggest

direct involvement of the sulfhydryl group. If this substitution, however, alters the affinity and/or selectivity of binding without eliminating it, it is concluded that other structural or chemical features of this amino acid are important in creating the native steroid-binding site.

For each of the positions a series of mutants are made. The replacements have progressively smaller side chains: 1) Trp to Tyr (or Phe) to Val to Ala to Gly; and 2) Cys to Ser to Ala to Gly. This strategy avoids disruption of tertiary structure interactions that could occur by introduction of a bulkier or charged side chain.

To alter the Cys and Trp residues in CBG in a predictable manner, the Inouye method as described in Morinaga et al., Bio. Tech., 2:639 (1986), which is incorporated herein by reference, for oligonucleotide-directed mutagenesis is used. Mutagenic oligonucleotides consist of the altered codon, flanked on either side by 10-15 nucleotides of cognate CBG sequence. For example, to change Cys_{228} to Ser, the mutagenic oligonucleotide has the sequence:

5′-GAC TCA GAG CTC CCG AGC CAG CTG GTG CAG-3′
Asp Ser Glu Leu Pro_{227} Ser_{228} Gln Leu Val Gln

Briefly, a first sample of pGEM plasmid containing CBG cDNA is digested with restriction endonuclease(s) flanking the region to be mutagenized. A second sample of the pGEM plasmid containing CBG cDNA is digested with a restriction endonuclease that cleaves at a site not found within the region flanked by the restriction endonucleases used in the first sample. The digested first and second plasmid samples are mixed, denatured in the presence of the mutagenic oligonucleotide, and reannealed to form gapped heteroduplexes so as to allow the oligonucleotide to anneal to the single-stranded region, and DNA polymerase and DNA ligase are used to

fill in the remaining single-stranded gaps and ligate the free ends of DNA. This results in heteroduplex plasmids containing one wild-type and one mutant strand. This DNA is then used to transform *E. coli*, and bacterial colonies containing the mutant sequence are identified as described below. A secondary transformation and screening is then performed to isolate mutant plasmids free of wild-type DNA. This approach has the advantage of allowing mutagenesis to be performed directly in the pGEM vector, and it eliminates further subcloning.

To identify mutant clones, creation or destruction of a restriction endonuclease recognition site in the course of introducing the desired mutation facilitates screening for the mutant. For instance, in the example shown above, the Pro_{227} codon is changed to CCG in the mutant from CCC in the wild-type. At the same time, the adjacent Cys_{228} codon, TGC, is changed to AGC to code for Ser (AGC). These changes create a new Ava1 restriction endonuclease site, 5'CCCGAG3'. This considerably facilitates the screening procedure by allowing the restriction endonuclease analysis of pools of plasmid DNA. If this approach is not feasible, mutant clones can be identified using a colony hybridization protocol in which the labelled mutagenic oligonucleotide is used as a probe. As a result of exact base-pairing, mutant:mutant hybrids are more resistant to temperature melting than mutant:wild type hybrids. Thus, washing the filters at increasing temperatures allows ready identification of the mutant DNAs.

Following identification of mutant plasmids, the mutants are confirmed by DNA sequencing, using the Sequenase method, which can be performed directly on intact pGEM plasmids. These plasmids are then be transcribed *in vitro*, and the mutant proteins

synthesized in the rabbit reticulocyte lysate system for initial testing. Finally, the mutant cDNAs are excised from the pGEM vector, and cloned into pVL1393 for incorporation into recombinant baculovirus.

While the foregoing discloses certain specific examples and techniques, the invention is not limited to the materials and methods disclosed, and variations in the specific materials and methods employed may be made without departing from the scope of the invention.

Claims

1. A chimeric protein comprising
   (a) an active steroid-binding moiety derived from corticosteroid binding globulin; and
   (b) a cell-binding moiety having a peptide sequence effective in specifically recognizing and binding to a target cell population different from the target cell population of native corticosteroid binding globulin.

2. A chimeric protein according to claim 1, wherein the active steroid-binding moiety comprises at least amino acids 228 through 371 of native human corticosteroid binding globulin.

3. A chimeric protein according to claim 1, wherein the cell-binding moiety is selected to bind to a cell population selected from the group consisting of $CD4^+$ lymphocytes and lymphocytes that contain cell-surface antibodies to myelin basic protein.

4. A cell chimeric protein according to claim 1, wherein the cell-binding moiety comprises a portion of the gp120 peptide of human immunodeficiency virus effective to allow the chimeric protein to specifically recognize and bind to $CD4^+$ lymphocytes.

5. A cell chimeric protein according to claim 4, wherein the portion of the gp120 peptide is residues 363-511.

6. A chimeric protein according to claim 2, wherein the cell-binding moiety is selected to bind to a

cell population selected from the group consisting of $CD4^+$ lymphocytes and lymphocytes that contain cell-surface antibodies to myelin basic protein.

7. A cell chimeric protein according to claim 2, wherein the cell-binding moiety comprises a portion of the gp120 peptide of human immunodeficiency virus effective to allow the chimeric protein to specifically recognize and bind to $CD4^+$ lymphocytes.

8. A cell chimeric protein according to claim 7, wherein the portion of the gp120 peptide is residues 363-511.

9. A pharmaceutical composition for delivery of a glucocorticoid to a target cell population in a patient, comprising

(a) a substantially purified protein comprising an active steroid-binding moiety derived from corticosteroid binding globulin and a cell-binding moiety having a peptide sequence effective to specifically recognize and bind to the target cell population;

(b) a glucocorticoid bound to the steroid-binding moiety of the protein; and

(c) a pharmaceutically acceptable carrier.

10. A composition according to claim 9, wherein the active steroid-binding moiety comprises at least amino acids 228 through 371 of native human corticosteroid binding globulin.

11. A composition according to claim 9, wherein the protein is a chimeric protein and the cell-binding moiety specifically recognizes and binds to a

target cell population different from the target cell population of native corticosteroid binding globulin.

12. A composition according to claim 11, wherein the active steroid-binding moiety comprises at least amino acids 228 through 371 of native human corticosteroid binding globulin.

13. A composition according to claim 11, wherein the cell-binding moiety is selected to bind to a cell population selected from the group consisting of $CD4^+$ lymphocytes and lymphocytes that contain cell-surface antibodies to myelin basic protein.

14. A composition according to claim 11, wherein the cell-binding moiety comprises a portion of the gp120 peptide of human immunodeficiency virus effective to allow the chimeric protein to specifically recognize and bind to $CD4^+$ lymphocytes.

15. A composition according to claim 14, wherein the portion of the gp120 peptide is residues 363-511.

16. A method for supplying a pharmacologically effective glucocorticoid to a targeted cell population in a patient comprising administering to the patient a pharmaceutical composition comprising

(a) a substantially purified protein comprising an active steroid-binding moiety derived from corticosteroid binding globulin and a cell-binding moiety having a peptide sequence effective to specifically recognize and bind to the target cell population;

(b) a glucocorticoid bound to the steroid-binding moiety of the protein; and

(c) a pharmaceutically acceptable carrier.

17. A method according to claim 16, wherein the active steroid-binding moiety comprises at least amino acids 228 through 371 of native human corticosteroid binding globulin.

18. A method according to claim 16, wherein the protein is a chimeric protein and the cell-binding moiety specifically recognizes and binds to a target cell population different from the target cell population of native corticosteroid binding globulin.

19. A method according to claim 18, wherein the active steroid-binding moiety comprises at least amino acids 228 through 371 of native human corticosteroid binding globulin.

20. A method according to claim 18, wherein the cell-binding moiety is selected to bind to a cell population selected from the group consisting of $CD4^+$ lymphocytes and lymphocytes that contain cell-surface antibodies to myelin basic protein.

21. A method according to claim 18, wherein the cell-binding moiety comprises a portion of the gp120 peptide of human immunodeficiency virus effective to allow the chimeric protein to specifically recognize and bind to $CD4^+$ lymphocytes.

22. A method according to claim 21, wherein the portion of the gp120 peptide is residues 363-511.

23. A plasmid comprising
(a) a sequence coding for an active steroid-binding moiety derived from corticosteroid binding globulin, and
(b) a promoter sequence recognized by insect cells, wherein the promoter sequence is effective to control the expression of the sequence coding for an active steroid-binding moiety.

24. A plasmid according to claim 23, wherein the promoter is the baculovirus polyhedrin promoter.

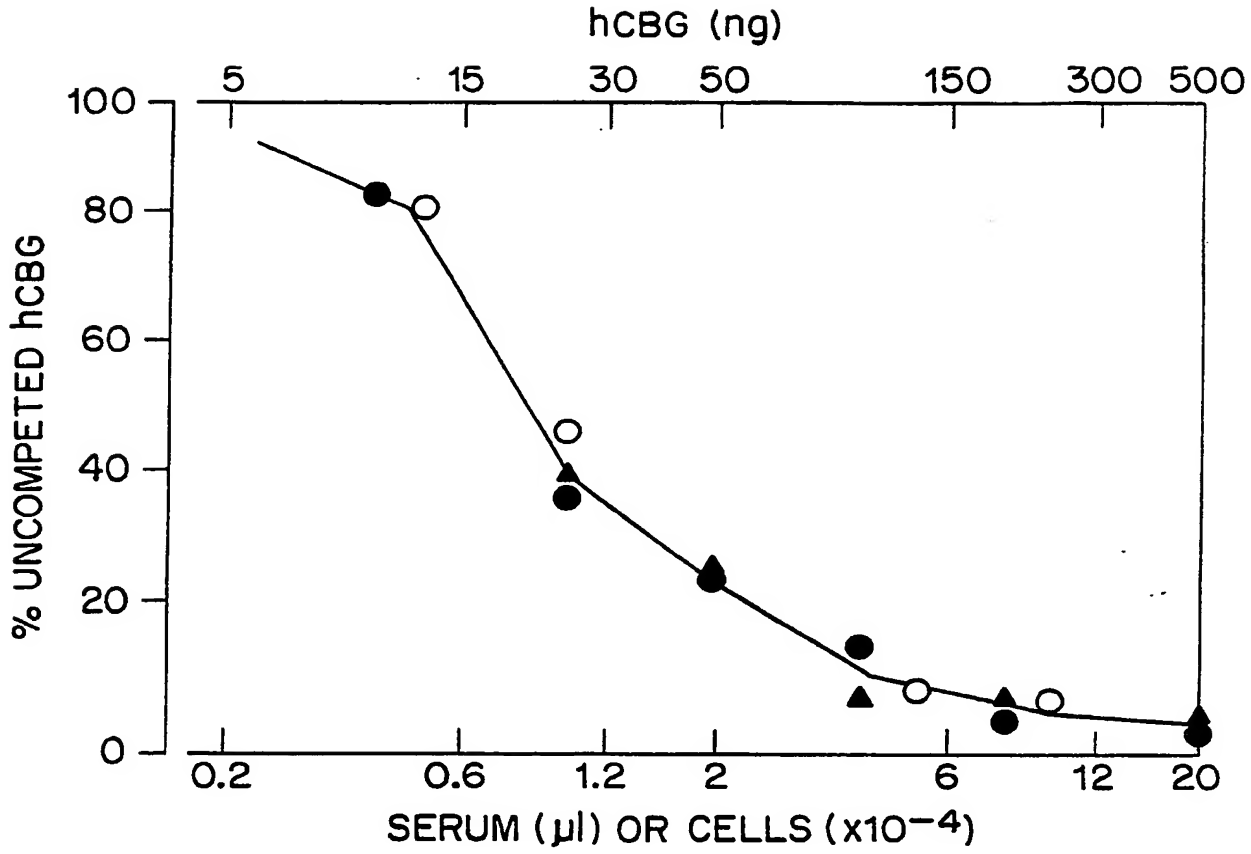
25. A plasmid according to claim 23, further comprising a sequence coding for a cell binding moiety having a peptide sequence effective to recognize and specifically bind to a target cell population wherein the sequence coding for the cell-binding moiety is under the control of the promoter.

26. A plasmid according to claim 25, wherein the promoter is the baculovirus polyhedrin promoter.

27. A plasmid according to claim 25, wherein the cell-binding moiety is selected from the group consisting of $CD4^+$ lymphocytes and lymphocytes that contain cell-surface antibodies to myelin basic protein.

28. A plasmid according to claim 27, wherein the cell-binding moiety corresponds to residues 363-511 of the gp120 peptide of human immunodeficiency virus.

29. A plasmid according to claim 28, wherein the promoter is the baculovirus polyhedrin promoter.


hCBG (ng)
5
15
30
50
150
300
500
100
80
60
40
20
0
% UNCOMPETED hCBG
0.2
0.6
1.2
2
6
12
20
SERUM (µl) OR CELLS (x10⁻⁴)